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Nanometric features of myosin filaments extracted from a single muscle fiber to uncover the mechanisms underlying organized motility

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ABSTRACT

The single muscle fiber *in vitro* motility assay (SF-IVMA) is characterized by organized linear motility of actin filaments, i.e., actin filaments motility showing a parallel or anti-parallel direction with similar speed independent of direction in the central part of the flow-cell where density of myosin is high. In contrast, the low myosin density region in the flow-cell exhibits random filament movements, but the mechanisms underlying the organized motility remain unknown. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) imaging techniques have been combined to investigate the morphological features of myosin extracted from single muscle fiber segments in the flow cell. Nanometric scale imaging of myosin filaments in the SF-IVMA showed intact spatial distances between myosin heads being essential for myosin filament function. However, angular spectrum analyses of myosin filaments in the high myosin density region showed organized myosin filament orientation only in small areas, while unorganized filament orientation were dominantly presented when larger areas were analyzed. Thus, parallel myosin filament organization is a less likely mechanism underlying the organized motility of actin filaments and the high myosin density *per se* is therefore forwarded as the primary "driver" that promotes organized linear motility.

1. Introduction

The single muscle fiber *in vitro* motility assay (SF-IVMA) has significant advantages over traditional *in vitro* motility assays, such as (i) the speed and force generated by myosin extracted from short muscle fiber sections (1–2 mm) can be measured with high precision [1,2], and (ii) the myofibrillar isoform expression is known in the fiber segments allowing detailed analyses of the function of specific myosin isoforms, myosin mutations and post-translational modifications of myosin [1–5]. The organized motility of actin filaments in the central region of the flow cell with high myosin density is a key feature of the SF-IVMA developed in our group, and organized motility is a criterion for acceptance since motility speed is less variable than unorganized randomly moving filaments [1]. However, the molecular mechanism underlying organized motility remains unknown. We have previously hypothesized that myosin extracted in high-salt solution polymerizes into myosin filaments in low-salt solution, attach to the glass or mica surface of the flow cell after flushing and organize parallel with the direction of the flow in a myosin high density streak [2,3]. In this study, we aim to investigate in detail the mechanism underlying the organized motility by using transmission electron microscopy (TEM) and atomic force microscopy (AFM) to examine the nanostructure of the myosin filaments in the high density region in the flow cell.

Based on the principle that electrons travel inside the sample, TEM imaging identifies the morphological features in internal or subcellular ultrafine structures [6,7]. In order to determine the

Abbreviations: SF-IVMA, single muscle fiber *in vitro* motility assay; TEM, transmission electron microscopy; AFM, atomic force microscopy; RH–PH, Rhodamine–phalloidin; SPIP, scanning probe image processor software; FFT, Fast Fourier Transformation; Stdi, texture direction index.

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surface topology of biological materials in their native states in near physiological buffer solutions [8–10], AFM was applied to provide detailed topographical mapping by raster scanning a fine tip gently over the surface of objects, resulting in a three dimensional profile with nanometric resolution [11]. More importantly, AFM does not require the sample to undergo any chemical treatment such as fixation or staining using heavy metal, or immunological modifications, thereby, eliminating preparation artefacts.

By combining TEM and AFM to investigate the ultramorphological features of myosin filaments extracted from the SF-IVMA, organized parallel arrangement of myosin filaments were only observed in small areas in the myosin high density region while organized motility was presented in large areas in the same region. Thus, parallel arrangement of myosin filaments in the myosin high density regions is an unlikely mechanism underlying the organized motility. In contrast to our original hypothesis, the high myosin density *per se* is forwarded as the dominant factor underlying the organized motility in the SF-IVMA.

2. Materials and methods

2.1. Muscle biopsies and bundle preparation

Soleus muscles were dissected from euthanized (removal of heart during deep isoflurane anesthesia) Sprague–Dawley rats and bundles of approximately 50 fibers were separated in relaxing solution at 4 °C. The bundles were tied to glass capillaries, stretched to about 110% of resting slack length, chemically skinned for 24 h at 4 °C in a relaxing solution containing 50% (vol/vol) glycerol, and kept at −20 °C for short-term storage. Within 1 week after skinning, the bundles were cryoprotected by transferring, at 30 min intervals, to relaxing solutions containing increasing concentrations of sucrose, (0, 0.5, 1.0, 1.5, and 2.0 M) [12], then frozen in liquid propane chilled by liquid nitrogen and stored at −160 °C. The day before an experiment, a bundle was transferred to a 2.0 M sucrose solution for 30 min and then incubated in solutions of decreasing sucrose concentration (1.5–0.5 M). The bundle was then stored in skinning solution at −20 °C. The study was approved by the ethical committees at Uppsala University and University of Turku.

2.2. Extraction of myosin from the single fiber

A short muscle fiber segment (1–2 mm) was placed on a glass slide between two strips of grease, and a glass coverslip or freshly cleaved mica plate (for TEM and AFM) placed on top, creating a flow cell of ~2 μl. Myosin was extracted from the fiber segment through addition of a high-salt buffer (0.5 M KCl, 25 mM Hepes, 4 mM $MgCl_2$, 4 mM EGTA and the pH value was adjusted to 7.6 before adding 2 mM ATP and 1% β-mercaptoethanol). After 30 min incubation on ice, a low-salt buffer (25 mM KCl, 25 mM Hepes, 4 mM $MgCl_2$, 1 mM EGTA and the pH value was adjusted to 7.6 before adding 1% β-mercaptoethanol) was applied. The pH of the buffers was adjusted with KOH, and the final ionic strength of the motility buffer was 71 mM.

2.3. Distribution of extracted myosin and direction of moving filaments

After rhodamine-phalloidin (RH-PH, Invitrogen, Carlsbad, CA, USA) labeled actin was incubated with extracted myosin, the fluorescence strip was imaged from the muscle fiber and the down-stream in the flow cell. When the motility was successfully established, the direction of moving filaments relative to the *x*-axis, in both central and outer regions was analyzed in the same preparation.

2.4. Preparation of grids with extracted myosin and TEM imaging and processing

The grids (200 mesh Ni) with Carbon-coated Formvar film were prepared. Then the grids were treated with 1% Alcian blue in water to make them hydrophilic. After air-drying, the grids were gently placed on the chamber with film side down onto the surface of glass slide, and the position was near to the end of muscle fiber segment in the myosin high density region. After successful motility, the cover slip was carefully removed, and the grid was picked up with anti-capillary tweezers and negatively stained by 1% Uranyl acetate by putting 3 drops of the solution on a piece of parafilm. Excess solution was then wiped off with filter paper and air dried thoroughly. The grids with the samples were examined by a Hitachi H 7100 electron microscope (Hitachi, Tokyo, Japan.). Electron micrographs were taken with a Gatan multiscan camera model 832 with Gatan digital micrograph software (Gatan, Pleasanton, CA, USA). In the data analysis procedure, prior to measurements, TEM images were processed with background subtraction in ImageJ with rolling ball size of 100 pixels, the intensity values were inverted, and the data were mean-filtered with 5 pixels in Gwyddion (v. 2.28 David Nečas and Petr Klapetek, Department of Nanometrology, Czech Metrology Institute).

2.5. Preparation of mica cover slip with extracted myosin and AFM imaging and image processing

The myosin fibrils were extracted directly onto freshly cleaved mica. After the extraction of myosin, the mica cover slip was removed from the flow chamber, and excess solution on the surface was removed gently. Fiber position was marked for AFM imaging. The mica cover slip was kept in a petri dish until it was imaged. Considering that long time non-hydrated environment would modify the conformation of the biomolecules, the AFM imaging was performed as soon as possible after finishing the preparation. The AFM imaging was performed with NTEGRA Prima scanning probe microscope (NT-MDT, Russia) and Agilent AFM 5500ILM (Agilent, USA). Surface topography was measured with intermittent contact mode under ambient conditions ($T = 24–25$ °C, RH = 17–34%) using uncoated rectangular silicon cantilevers (MikroMash, model NSC14/NoAl, NSC15/NoAl or NSC19/NoAl). Images were recorded in the repulsive regime using a damping ratio of 0.7 and a scan speed of 0.25–0.50 Hz. The images were processed with scanning probe image processor software (SPIP version 6.0.6 Metrology A/S, Denmark or Gwyddion 2.28). The central part of the mica cover slip close to the end of muscle fiber segment was imaged.

AFM images were pre-processed for image analysis. For the analysis of myosin filament size, two basic functions in Gwyddion, plane level and median line corrections were used. For the angular spectrum analysis, global levelling and linewise bow removal functions, long-pass − filtering with a parameter of 1/6th of the frame size were used for leveling. In addition, the image was first transformed into frequency domain by Fast Fourier Transformation (FFT), processed with low pass filter with a wavelength of 8 for high frequency noise removal, and reconstructed with inverse FFT. For the angular spectrum analysis, the roughness calculation function in SPIP software was used. The analysis provides an angle of the cross-section along which the highest amplitude (Std) is recorded, and a texture direction index (Stdi) measuring dominance of certain Std over the area measured, i.e., Std in the angular spectrum reaches its highest value at an angle perpendicular to the texture direction. The Stdi value indicates relative dominance of angular spectrum between